REMARKS

Claim status. Claims 1 to 7, 63, and 64 remain pending. Claims 8 to 62 were canceled in a previous amendment.

Rejection under Section 112. The Examiner maintained the rejection from the previous office action over the Applicants' arguments based on *Enzo v. Gen-Probe*, 63 U.S.P.Q. 2d 1609 (Fed. Cir. 2002). The Applicants maintain their argument that the presently claimed Fc-peptide fusion molecules are analogous to the antibodies discussed in *Enzo*.

The Examiner's argument appears to be based on a few misconceptions. First, the Examiner stated that "it is not seen how a random peptide is considered a well-defined structure when it is a random sequence." (Office Action at page 6). The Applicants do not contend that the *peptides* are a well-defined structure, but rather that the Fc molecules defined by the structural formula (now referred to as *peptibodies*) provide a structure that, together with the functional characteristic, satisfies the written description requirement. It is well known in the art that an antibody possesses not only constant regions but also variable and highly variable regions. Despite this, the *Enzo* court held that an antibody claim without sequence information in the specification satisfies the written description requirement. Likewise, the current peptibody claim provides a structure coupled with a functional parameter and thus satisfies the written description requirement. See *Enzo*, cited above.

Second, the Examiner noted the part of the definition of "randomized" referring to replacement of residues in known sequences and alleged that "there is not a single known sequence for said sequence to be randomized let alone its binding to Ang-2." (Office Action at page 6). The Applicants call the Examiner's attention to the known receptor Tie-2, which does bind to Ang-2. See, for example, Kim et al. (2000), *Biochem J.* 346: 603-610 (copy enclosed).

Third, the Examiner argued that the claimed molecule was a "complex comprising known and unknown components." The peptide portion, however, is defined by its functional characteristic (ang-2 binding), just as the variable domain of an antibody, including the highly variable complementarity determining regions (CDRs), is defined by a functional characteristic. The Examiner's rationale would reject an antibody claim that the *Enzo* court would find to have sufficient written description.

Fourth, the Examiner argued that claims 3 and 4 fail to provide written description because they recite "products by processes" (emphasis in original) and that "it is not evident from the disclosure which method from the numerously recited ones has been employed that results in a peptide that binds to Ang-2" (Office Action at page 7). The inclusion of multiple alternatives for any step in a process, however, is irrelevant; the Examiner cites no authority for rejecting the claims on this basis. Furthermore, the specification clearly alleges that each of the

recited processes may be employed. Further still, the Examiner's use of the past tense ("has been employed") ignores the possibility of constructive reduction to practice.

Fifth, the Examiner argued that the holding in the *Eli Lilly* case was applicable in this instance. In *Lilly,* however, the court dealt with a claim to a "mammalian" protein when only one species was described. In that case, the patentee provided no description of a common characteristic--functional or structural--and no description of how to obtain the orthologs in other species. Here, in contrast, the Applicants clearly describe a common structure (the Fc domain), a common functional characteristic (ang-2 binding), and methods for preparing the claimed molecule (e.g., using phage display). The Examiner further noted that the *Lilly* court cited structure, formula and chemical name as examples of suitable written description. The court did, however, recite these as *examples*—the court did not intend to exclude any other form of written description. The later *Enzo* case is consistent with *Lilly* in providing additional means for complying with the written description requirement.

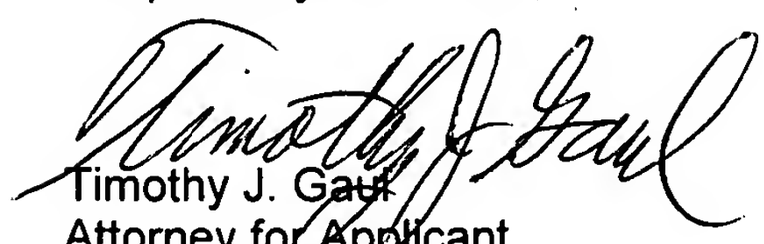
Rejection under Section 103. As in the rejection under Section 112, the Examiner's arguments appear to be based on misconceptions. First, the Examiner argued that the mention of degenerate sequences in Cerretti et al. suggested randomization, stating, "In reply, degeneracy of genetic code do not maintain the encoded amino acid sequence." (Office Action at page 9). That statement is incorrect. See enclosure from Lewin, *Genes IV*, Oxford: Oxford University Press, 1990.

Second, the Examiner argued that the applicants did not specifically point out where in the specification the randomized ang-2 binding peptides are described. As noted above, however, the Applicants do not allege that actual reduction to practice is described in the specification. The specification provides numerous examples of peptide selection and explicitly identifies ang-2 as a molecule to be used in peptide selection. The patent law does not require actual reduction to practice.

Third, the Examiner argued that Cerretti *et al.* render the claims obvious because Tek antibodies allow a variation of up to 10 residues and that, since the Applicants do not preclude the presence of other sequences attached to the ang-2 binding peptide, the Applicants' claim would include the Tek antibody. This argument ignores the fact that Tek antibodies bind to Tek, not ang-2. There is nothing in Cerretti *et al.* suggesting that its variation of 10 residues would generate a region that binds to ang-2.

Conclusion. In light of the foregoing remarks, the Applicants respectfully request reconsideration of the Office Action, entry of all amendments, and allowance of all claims.

Respectfully submitted,



Timothy J. Gaul
Attorney for Applicant
Registration No.: 33,111
Phone: (805) 447-2688
Date: June 30, 2005

Please send all future correspondence to:
U.S. Patent Operations/ TJG
Dept. 430, M/S 27-4-A
AMGEN INC.
One Amgen Center Drive
Thousand Oaks, California 91320-1799

genes to evolve together as though constituting a single locus.

Condensation reaction is one in which a covalent bond is formed with loss of a water molecule, as in the addition of an amino acid to a polypeptide chain.

Conditional lethal mutations kill a cell or virus under certain (nonpermissive) conditions, but allow it to survive under other (permissive) conditions.

Conjugation describes "mating" between two bacterial cells, when (part of) the chromosome is transferred from one to the other.

Consensus sequence is an idealized sequence in which each position represents the base most often found when many actual sequences are compared.

Conservative recombination involves breakage and reunion of preexisting strands of DNA without any synthesis of new stretches of DNA.

Constant regions of immunoglobulins are coded by C genes and are the parts of the chain that vary least. Those of heavy chains identify the type of immunoglobulin.

Constitutive genes are expressed as a function of the interaction of RNA polymerase with the promoter, without additional regulation; sometimes also called household genes in the context of describing functions expressed in all cells at a low level.

Constitutive heterochromatin describes the inert state of permanently nonexpressed sequences, usually satellite DNA.

Constitutive mutations cause genes that usually are regulated to be expressed without regulation.

Contractile ring is a ring of actin filaments that forms around the equator at the end of mitosis and is responsible for pinching the daughter cells apart.

Controlling elements of maize are transposable units originally identified solely by their genetic properties. They may be autonomous (able to transpose independently) or nonautonomous (able to transpose only in the presence of an autonomous element).

Coordinate regulation refers to the common control of a group of genes.

Cordycepin is 3′ deoxyadenosine, an inhibitor of polyadenylation of RNA.

Core DNA is the 146 bp of DNA contained on a core particle.

Core particle is a digestion product of the nucleosome that retains the histone octamer and has 146 bp of DNA; its structure appears similar to that of the nucleosome itself.

Corepressor is a small molecule that triggers repression of transcription by binding to a regulator protein.

Cosmids are plasmids into which phage lambda *cos* sites have been inserted; as a result, the plasmid DNA can be packaged *in vitro* in the phage coat.

Cot is the product of DNA concentration and time of incubation in a reassociation reaction.

$Cot_{1/2}$ is the Cot required to proceed to half completion of the reaction; it is directly proportional to the unique length of reassociating DNA.

Cotransfection is the simultaneous transfection of two markers.

Crossing-over describes the reciprocal exchange of material between chromosomes that occurs during meiosis and is responsible for genetic recombination.

Crossover fixation refers to a possible consequence of unequal crossing-over that allows a mutation in one member of a tandem cluster to spread through the whole cluster (or to be eliminated).

Cruciform is the structure produced at inverted repeats of DNA if the repeated sequence pairs with its complement on the same strand (instead of with its regular partner in the other strand of the duplex).

Cryptic satellite is a satellite DNA sequence not identified as such by a separate peak on a density gradient; that is, it remains present in main-band DNA.

ctDNA is chloroplast DNA.

Cyclic AMP (cAMP) is a molecule of AMP in which the phosphate group is joined to both the 3′ and 5′ positions of the ribose; its binding activates the CAP, a positive regulator of prokaryotic transcription.

Cytokinesis is the final process involved in separation and movement apart of daughter cells at the end of mitosis.

Cytological hybridization—*see in situ* hybridization.

Cytoplasm describes the material between the plasma membrane and the nucleus.

Cytoplasmic inheritance is a property of genes located in mitochondria or chloroplasts (or possibly other extranuclear organelles).

Cytoplasmic protein synthesis is the translation of mRNAs representing nuclear genes; it occurs via ribosomes attached to the cytoskeleton.

Cytoskeleton consists of networks of fibers in the cytoplasm of the eukaryotic cell.

Cytosol describes the general volume of cytoplasm in which organelles (such as the mitochondria) are located.

D loop is a region within mitochondrial DNA in which a short stretch of RNA is paired with one strand of DNA, displacing the original partner DNA strand in this region. The same term is used also to describe the displacement of a region of one strand of duplex DNA by a single-stranded invader in the reaction catalyzed by RecA protein.

Degeneracy in the genetic code refers to the lack of an effect of many changes in the third base of the codon on the amino acid that is represented.

Deletions are generated by removal of a sequence of DNA, the regions on either side being joined together.

Denaturation of DNA or RNA describes its conversion from the double-stranded to the single-stranded state; separation of the strands is most often accomplished by heating.

Denaturation of protein describes its conversion from the

Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis

Injune KIM*, Hwan-Gyu KIM*, Hyun KIM‡, Hong-Hee KIM§, Sung Kwang PARK†, Chang-Sub UHM‡, Zang Hee LEE§ and Gou Young KOH*[1]

*National Creative Research Initiatives Center for Cardiac Regeneration and Institute of Cardiovascular Research, Chonbuk National University School of Medicine, Chonju, 560-180, Korea, †Department of Internal Medicine, Chonbuk National University School of Medicine, Chonju, 560-180, Korea, ‡Department of Anatomy, Institute of Human Genetics and Graduate School of Biotechnology, Korea University, Seoul, 136-705, Korea, and §Department of Microbiology and Immunology, Chosun University Dental School, Kwangju, 501-759, Korea

Using degenerate PCR we isolated a cDNA encoding a novel 406- and 410-amino acid protein from human and mouse embryonic cDNAs and have designated it 'hepatic fibrinogen/angiopoietin-related protein' (HFARP). The N-terminal and C-terminal portions of HFARP contain the characteristic coiled-coil domains and fibrinogen-like domains that are conserved in angiopoietins. In human and mouse tissues, HFARP mRNA is specifically expressed in the liver. HFARP mRNA and protein are mainly present in the hepatocytes. HFARP has a highly hydrophobic region at the N-terminus that is typical of a secretory signal sequence and one consensus glycosylation site. Recombinant HFARP expressed in COS-7 cells is secreted and glycosylated. HFARP protein is present not only in the hepatocytes, but also in the circulating blood. Recombinant HFARP acts as an apoptosis survival factor for vascular endothelial cells, but does not bind to Tie1 or Tie2 (endothelial-cell tyrosine kinase receptors). These results suggest that HFARP may exert a protective function on endothelial cells through an endocrine action.

Key words: endocrine; liver; Tie receptor.

INTRODUCTION

The fibrinogen protein superfamily includes the fibrinogens, the angiopoietins and the ficolins. Angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) have characteristic protein structures that contain coiled-coil domains in the N-terminal portion and fibrinogen-like domains in the C-terminal portion [1,2]. The discovery of Ang1 and Ang2 has provided insight into the molecular and cellular mechanisms of blood-vessel formation [1–3]. Ang1 and Ang2 share about 60 % amino acid identity and bind with similar affinity to the endothelial-cell tyrosine kinase receptor Tie2 [1,2].

Using homology-based PCR, we previously isolated a cDNA encoding a novel angiopoietin-related protein and designated it angiopoietin-3 (Ang3) [4]. However, Valenzuela et al. [5] recently isolated a novel subfamily of angiopoietins and named them mouse angiopoietin-3 (Ang3) and human angiopoietin-4 (Ang4); the two are probably interspecies orthologues. On the basis of amino acid similarity, their Ang3 and Ang4 are closer to Ang1 and Ang2 than our Ang3. Importantly, their Ang3 and Ang4 bound to the Tie2 receptor, but not to the Tie1 receptor. Therefore, we rename our previously isolated Ang3 as angiopoietin-related protein-1 (ARP1). In addition, we isolated the cDNA encoding other novel angiopoietin-related protein (ARP) and have designated it angiopoietin-related protein-2 (ARP2), since the amino acid sequence of ARP2 is closest to that of ARP1 [6]. ARP1 and ARP2 also have characteristic protein structures that contain coiled-coil domains in the N-terminal portion and fibrinogen-like domains in the C-terminal portion [4,6]. ARP1 and ARP2 share 59 % amino acid identity. Notably, ARP2 was preferentially expressed in the blood vessels and skeletal muscles of developing rat embryos. ARP2 is a glycosylated secretory protein that induces sprouting in endothelial cells, probably through an autocrine or paracrine activity [6].

In the present study we isolated a cDNA encoding another novel fibrinogen/angiopoietin-related protein and have designated it 'hepatic fibrinogen/angiopoietin-related protein' (HFARP) because its expression is predominantly in the hepatocytes of embryo and adult. HFARP is a glycosylated secretory protein that is an apoptosis survival factor in endothelial cells, probably through an endocrine activity.

MATERIALS AND METHODS

Isolation of human and mouse HFARP

Partial cDNAs of human and mouse HFARP were amplified using human and mouse embryonic cDNAs (ClonTech, Palo Alto, CA, U.S.A.) as PCR templates. PCR was performed for 30 cycles at an annealing temperature of 52 °C using sense and antisense degenerate primers representing all possible codons for

Abbreviations used: HFARP, hepatic fibrinogen/angiopoietin-related protein; Ang, angiopoietin; ARP, angiopoietin-related protein; RACE, rapid amplification of cDNA ends; HUVECs, human umbilical-vein endothelial cells; PPAECs, porcine pulmonary arterial endothelial cells; E, embryonic day; CMV, cytomegalovirus; $VEGF_{165}$, vascular endothelial growth $factor_{165}$; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; hFREP-1, human fibrinogen-related protein 1; pFicloinB, porcine ficolin β; hpFGA, human fibrinogen A polypeptide; PNGase-F, peptide N-glycosidase F; MC, microcarrier.

[1] To whom correspondence should be addressed (e-mail gykoh@moak.chonbuk.ac.kr).

The nucleotide sequences for the human and mouse hepatic angiopoietin-related protein genes have been deposited in the GenBank®, DDBJ and EMBL Nucleotide Sequence Databases under the accession numbers AF169312 and AF169313.

the following peptides of human Ang1 and Ang2: GEYWLG (Ang1, amino acids 353–358; Ang2, amino acids 351–356) and SNLNGM (Ang1, amino acids 455–460; Ang2, amino acids 453–458) [1,2]. A DNA band of expected size (≈ 300 bp) was amplified. The amplified DNA was sequenced by cycle sequencing using the AmpliCycle sequencing kit (Perkin–Elmer Corp., Foster City, CA, U.S.A.). The novel amplified DNA was cloned into the pCR-Blunt vector (Invitrogen, San Diego, CA, U.S.A.). To clone the remaining coding region, human and mouse embryonic cDNAs were used for rapid amplification of cDNA ends (RACE; ClonTech).

Cell culture

Human umbilical-vein endothelial cells (HUVECs) and porcine pulmonary arterial endothelial cells (PPAECs) were prepared from human umbilical cords and porcine pulmonary arteries by collagenase digestion. The endothelial origin of the cultures was confirmed by the presence of von Willebrand factor detected by immunofluoresence. HUVECs and PPAECs were maintained in M-199 medium supplemented with 20% (v/v) fetal-bovine serum. COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% (v/v) fetal-bovine serum at 37 °C in a 5% CO_2 atmosphere. The primary cultured cells used in this study were between passages 2 and 4.

Northern-blot analysis

A ^{32}P-labelled human HFARP cDNA probe (nts 16–339) and a ^{32}P-labelled mouse HFARP cDNA probe (nts 1–591) were radiolabelled by random priming (Prime-a-Gene; Promega, Madison, WI, U.S.A.). The human probe was hybridized to a human multiple tissue Northern blot (ClonTech) according to the manufacturer's instructions. RNA extractions and Northern-blot analysis were performed as previously described [7].

In situ hybridization in rat embryo

In situ hybridization was performed essentially as previously described [8]. In brief, 18-day-old embryos (E18) and adult liver of Harlan Sprague–Dawley rat were frozen in prechilled isopentane. Sagittal and frontal sections (12 μm thick) were cut and thaw-mounted on to gelatin-coated slides. The sections were fixed, treated with acetic anhydride, dehydrated, and finally air-dried. An ^{35}S-labelled mouse HFARP antisense (nts 1–591) cRNA probe was transcribed using the Riboprobe (Promega) with [α-^{35}S]UTP (Amersham Pharmacia Biotech). Sections were hybridized overnight with labelled probe, treated with RNAse A, washed sequentially in 0.15 M NaCl/0.015 M sodium citrate ('SSC'), briefly rinsed in a graded series of ethanol solutions and dried. Slides were placed in X-ray cassettes, exposed to β-Max film (Amersham Pharmacia Biotech) for 5 days, and developed. Slides bearing hybridized tissue sections were dipped in NTB2 (Eastman Kodak Co., Rochester, NY, U.S.A.) emulsion, exposed for 2 weeks and counterstained with Cresyl Violet after development.

Antibody production, immunohistochemistry and Western-blot analysis

Polyclonal anti-HFARP antibody was produced by immunization of rabbit using standard methods. The antigen was a recombinant mid-portion of HFARP that was produced in *Escherichia coli*. The cDNA encoding the mid-portion of HFARP (nts 320–920) was inserted in the correct open reading frame into the glutathione S-transferase fusion protein expression vector pGEX4T-1 (Amersham Pharmacia Biotech). The expression of HFARP protein was induced in the presence of 0.1 mM isopropyl D-thiogalactopyranoside, and purified using a glutathione–Sepharose 4B column. The glutathione S-transferase protein was removed by thrombin treatment. The purified protein was mixed with an equal volume of complete or incomplete Freund's adjuvant and injected subcutaneously into the back neck area of a rabbit for initial and booster injections. Adult rat liver was fixed with 10% (v/v) neutral buffered formalin. Paraffin tissue blocks were sectioned at 4 μm. Sections were incubated with anti-HFARP antibody at 4 °C overnight, and signals were visualized with the EnVison (Dako, Glostrup, Denmark) system. Sections were then counterstained with Meyer's haematoxylin. Blood sampling was performed from abdominal aorta of normal male rat under pentobarbital sodium (30 mg/kg). Plasma was diluted with PBS, mixed with sample buffer, boiled for 10 min, separated by SDS/10%-PAGE under denaturing conditions, and electroblotted to nitrocellulose membranes. The nitrocellulose membranes were blocked by incubation in blocking buffer, incubated with anti-HFARP polyclonal antibody, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Signals were revealed by chemiluminescence detection according to the manufacturer's protocol [ECL® (enhanced chemiluminescence); Amersham Pharmacia Biotech].

Expression, purification and detection of recombinant proteins

Human HFARP cDNA was inserted into the cytomegalovirus (CMV) promoter-driven mammalian cell expression vector pcDNA3.1/Myc-His (Invitrogen). This vector contains a 63 bp *c*-Myc tag (EQKLISEEDL) and a His_6 tag (HHHHHH) as open reading frames at the 3′-terminus of the coding region. Each gene construct was transfected into COS-7 cells using LipofectAMINE Plus (Gibco BRL, Gaithersburg, MD, U.S.A.) and incubated at 37.5 °C for 72 h in Dulbecco's modified Eagle's medium with 2% (v/v) fetal-bovine serum under 5% CO_2. Ni^{2+}-nitrilotriacetate–agarose beads (Qiagen, Hilden, Germany) were added to the culture supernatant of the COS-7 cells to purify the recombinant proteins. All buffers contained 0.05% (w/v) CHAPS during protein purification and reconstitution. The binding protein was eluted with pH 8.0 buffer containing 250 mM imidazole. The eluates were dialysed extensively against 0.05 M Tris/HCl (pH 7.5)/150 mM NaCl (TBS buffer) and were concentrated using Centricon 10 (Amicon, Beverly, MA, U.S.A.) at 4 °C. The salts were removed using a desalting column and the proteins were reconstituted with TBS buffer. Approx. 50 μg of the recombinant protein was obtained. Ang1 and vascular endothelial growth $factor_{165}$ ($VEGF_{165}$) were obtained in the same manner as HFARP [6]. The purity of this protein was determined by silver staining after SDS/PAGE. The proteins were electroblotted to nitrocellulose membranes and confirmed again by Western-blot analysis with anti-Myc or anti-HFARP antibody as described above. The glycosylation status of the recombinant HFARP was determined with peptide N-glycosidase F treatment according to the manufacturer's protocol (New England Biolabs, Beverly, MA, U.S.A.).

Mitogenic, sprouting and anti-apoptosis assay of HFARP for endothelial cells

Mitogenic and sprouting assays in HUVECs and PPAECs were performed as previously described [4,9–11]. The effect of HFARP, Ang1 or fibrinogen (Sigma, St. Louis, MO, U.S.A.) on serum-deprivation-induced apoptosis in HUVECs was examined as described previously [11]. Briefly, HUVECs were plated on to gelatinized 24-well plates (5×10^4 cells/well) in M-199 medium

supplemented with 10% (v/v) fetal-bovine serum and incubated for 24 h. The wells were extensively washed with PBS, and the medium was changed to serum-free M-199 medium containing various reagents and incubated for 24 h. Apoptosis was quantified as described previously [11]. Floating cells were collected with two PBS washes; adherent cells were collected by trypsin treatment The numbers and size distributions of the floating and adherent cells were determined with a Coulter Model Z1 Dual Counter System. More than 95% of the floating cells were apoptotic cells, as confirmed by terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay kit (Oncor, Gaithersburg, MD, U.S.A.). To detect the apoptotic cells among the adherent cells, the cells in parallel wells were washed with PBS, fixed for 5 min with 1.0% (w/v) paraformaldehyde and subjected to a TUNEL assay. The adherent cells were examined at 400 × magnification, and the stained cells were counted in four different random locations, each containing approx. 250 cells. The percentage of apoptotic cells is based on the sum of the floating cells plus the apoptotic adherent cells in a given cell population.

In vitro binding assay between HFARP protein and extracellular domain of Tie1 or Tie2 receptor

The secreted extracellular domains of the Tie1 and Tie2 receptors were produced using pFLAG CMV-1 vector (Eastman Kodak Co.) which has a preprotrypsin leader sequence for secretion of the fusion protein. The extracellular domains of human Tie1 (amino acids 28–752) and Tie2 (amino acids 7–729) were cloned into pFLAG CMV-1 in an open reading frame. These constructs were transfected into COS-7 cells and the culture supernatants were collected for 48 h for the binding assay. These fusion proteins were designated as pFLAG-eTie1 and pFLAG-eTie2. Anti-FLAG M1 affinity gel (Eastman Kodak Co.) was used for holding pFLAG-eTie1 or pFLAG-eTie2 protein. The gels were washed with binding buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.4) and then the culture supernatant containing recombinant Ang1 or HFARP was added to this gel. After 1 h incubation, the gels were cleared with washing buffer [50 mM Tris/150 mM NaCl/1 mM $CaCl_2$ (pH 7.4)/0.05% (v/v) Tween-20]. The gels were boiled with sample buffer and the samples were separated by SDS/7.5%- or 10%-PAGE. Anti-FLAG-M2 antibody was used to detect the binding of the pFLAG-eTie1 and pFLAG-eTie2 proteins. Anti-Myc antibody was used to detect the binding of Ang1 or HFARP.

RESULTS

Cloning and analysis of human and mouse HFARP

We used degenerate PCR of a human embryonic cDNA to obtain a product with a novel sequence related to the angiopoietins. We used RACE PCR to clone the remaining coding region and designated the gene *HFARP*. The human HFARP cDNA encodes a 406-amino-acid polypeptide (Figure 1A). Mouse *HFARP* was cloned by the same methods and shares 75% nucleotide identity with the human gene. The 410-amino acid mouse protein shares 77% amino acid identity with the human HFARP product.

According to the ProteinPrediction program (EMBL), HFARP has coiled-coil domains in the N-terminal portion and conserved fibrinogen-like domains in the C-terminal portion, as do angiopoietins (Figure 1A). According to the results from BlastP (NCBI) and ClustalW programs, the C-terminal portion of HFARP is shared by a variety of other proteins, including the following: human Ang5 [12], Ang2 [2], Ang1 [1], Ang3 [5], Ang4

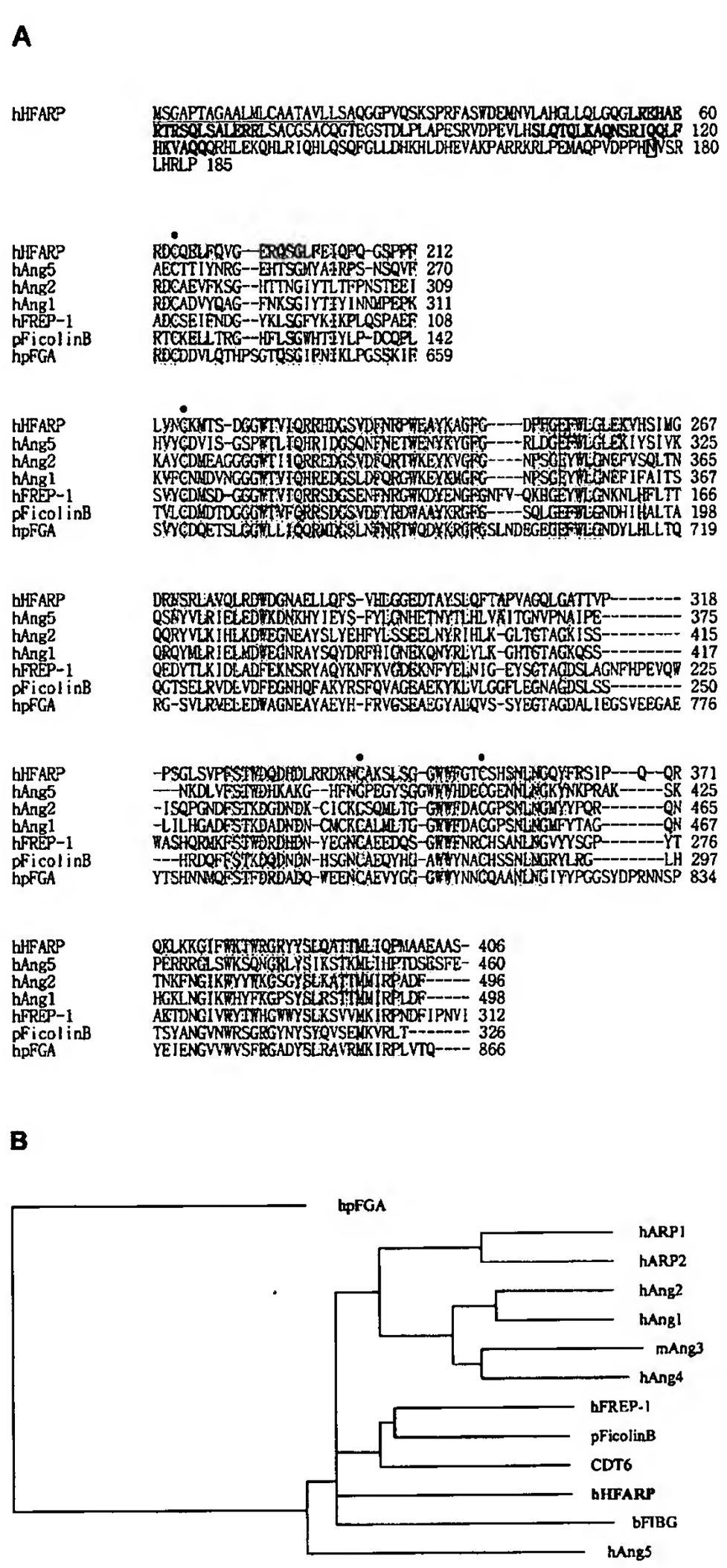


Figure 1 Comparison of amino acid sequences and neighbour-joining analysis of HFARP and its relatives

(**A**) Alignment of the deduced amino acid sequences of human HFARP with C-terminal portions (fibrinogen-like domains) of hAng5, human Ang2, human Ang1, hFREP-1, pFicolinB and hpFGA. Residues that match the sequence of human HFARP are shadowed. The line below the human HFARP sequence marks the putative secretory signal sequence [18]. Two coiled-coil domains in the N-terminal portions are emboldened. The square denotes a potential glycosylation site. The filled circles above the human HFARP sequence denote four cysteine residues that are conserved in angiopoietin family proteins. (**B**) Neighbour-joining tree of 13 proteins. Their relationship was analysed using the ClustalW program. The length of each horizontal line is proportional to the degree of amino acid sequence divergence.

[5], human fibrinogen-related protein-1 (hFREP-1) [13], CDT6 (angiopoietin-like factor from human cornea) [14], bovine fibrinogen γ-B chain precursor [15], porcine ficolin β (pFicloinB)

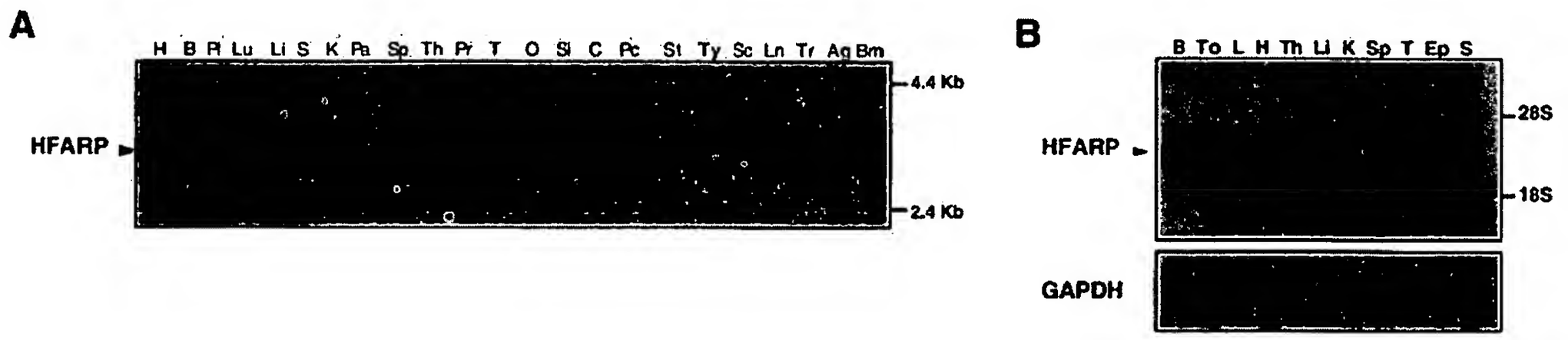


Figure 2 Northern-blot analysis (A) of HFARP mRNA in human adult tissues and (B) for detection of HFARP mRNA in adult mouse tissues

(**A**) Each lane contains approx. 2 μg of purified polyadenylated RNA from adult heart (H), brain (B), placenta (Pl), lung (Lu), liver (Li), skeletal muscle (S), kidney (K), pancreas (Pa), spleen (Sp), thymus (Th), prostate (Pr), testis (T), ovary (O), small intestine (Si), colon (C), peripheral-blood leukocyte (Pc), stomach (St), thyroid (Ty), spinal cord (Sc), lymph node (Ln), trachea (Tr), adrenal gland (Ag) and bone marrow (Bm). Hybridization was performed with a ^{32}P-labelled human HFARP probe. The sizes of the RNA molecular-size makers, in kb, are shown on the right. (**B**) Each lane contains 20 μg of total RNA from adult brain (B), tongue (To), lung (Lu), heart (H), thymus (Th), liver (Li), kidney (K), spleen (Sp), testis (T), epididymus (Ep) and skeletal muscle (S). Hybridization was performed with a ^{32}P-labelled mouse HFARP probe and the blot was re-hybridized with a ^{32}P-labelled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe to verify equal loading of RNA in each lane.

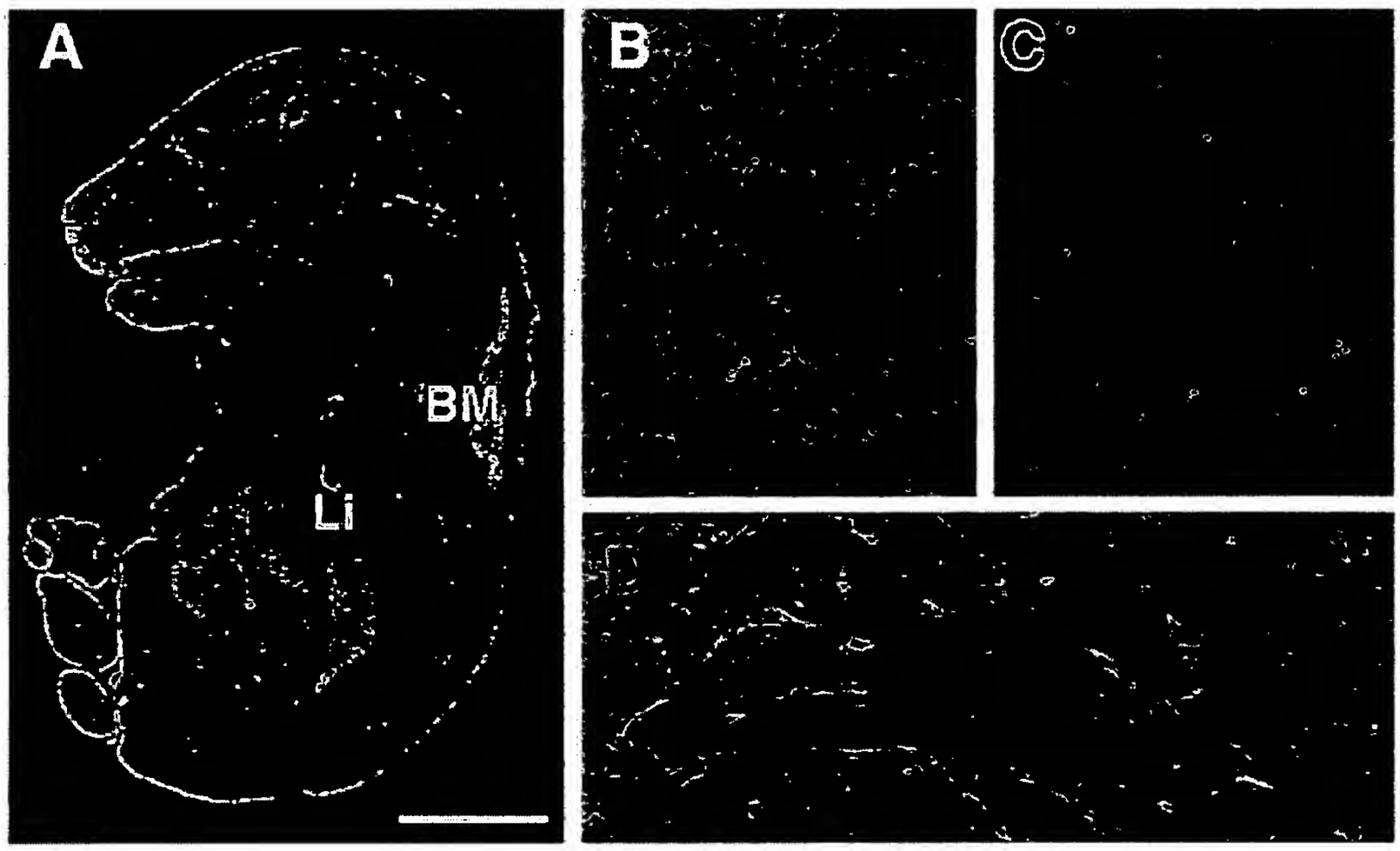


Figure 3 *In situ* hybridization of (A) HFARP mRNA and (B and C) HFARP mRNA in liver, and (D) immunohistochemistry of HFARP protein

(**A**) Autoradiograph shows the localization of HFARP ^{35}S-labelled riboprobe hybridized to sagittal section of 18-day-old embryo of the rat. L, liver; BM, back muscle. The scale bar represents 5 mm. (**B** and **C**) Bright-field photomicrographs show detectable silver black grains over hepatocyes with HARP ^{35}S-labelled riboprobe hybridized to rat embryonic liver (**B**) and adult liver (**C**). (**D**) Optical photomicrograph shows reddish-brown immunopositive signals in adult rat liver. Heterogenous pattern of HFARP immunopositive signals is observed in the cytoplasm of hepatocytes. Magnification 400 ×.

[16], and human fibrinogen A polypeptide (hpFGA) [17]. The alignment of the deduced amino acid sequence of HFARP with C-terminal portions of those of Ang5, Ang2, Angl, hFREP-1, pFicolinB and hpFGA is shown (Figure 1A). When gaps in the alignments are ignored in the calculations, the C-terminal portion of HFARP is 37–40 % identical with the C-terminal portion of Ang5, Ang2, Angl, hFREP-1, pFicloinB and hpFGA. In this region, four of the five cysteines found in all angiopoietins are conserved in HFARP [1]. The ClustalW program was used to produce a neighbour-joining tree to analyse the relationship between HFARP and its relatives (Figure 1B). HFARP is close to the fibrinogen, angiopoietin and ficolin protein families.

Expression HFARP mRNA and HFARP protein

Northern blotting of human and mouse adult tissues revealed that 3.4 kb of HFARP mRNAs was specifically expressed in the liver among most of tissues that were examined (Figure 2). The results seen by *in-situ*-hybridization analysis in rat embryo (E18) and adult liver were consistent with those of the Northern-blot

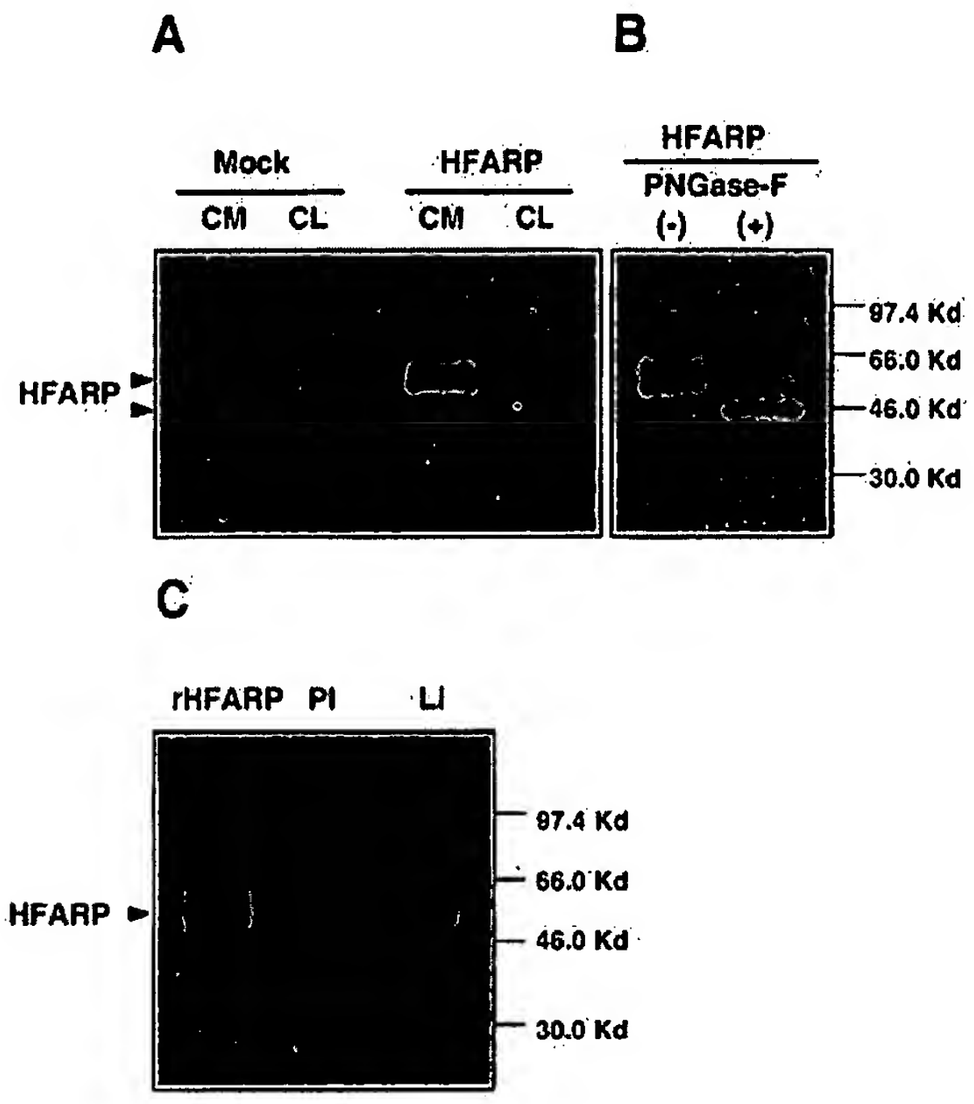


Figure 4 (A) *In vitro* expression of HFARP protein, (B) comparison of the molecular masses of recombinant HFARP protein with (+) or without (−) PNGase-F treatment, and (C) Western-blot analysis of HFARP protein in rat plasma

(**A**) Detection of human HFARP protein from the culture medium (CM) and cell lysates (CL) of COS-7 cells transfected with CMV promoter-driven mammalian cell expression vector only (Mock) or containing the human HFARP cDNA with the 3′-terminal extension encoding *c*-Myc and His_6 tags. The samples were separated by SDS/10%-PAGE, blotted, and probed with anti-Myc antibody. (**B**) Each lane contains 200 ng of purified human recombinant HARP protein, probed with anti-Myc antibody. (**C**) The samples were separated by SDS/10%-PAGE, blotted, and probed with anti-HFARP polyclonal antibody. rHFARP, 500 ng of recombinant HFARP protein; PI, 2 μl of plasma; LI, 100 μg of protein lysate of rat liver tissue. Molecular-mass-marker sizes shown were used to estimate molecular masses. Results were similar in three independent experiments. Kd, kilodaltons.

analyses (Figure 3). The HFARP mRNA transcripts were specifically abundant in the liver and back muscle of rat embryo (Figure 3A). Silver-grain staining indicated that HFARP mRNA was detected mainly in the hepatocytes, but not in the Kupffer cells, endothelial cells or blood vessels in embryonic and adult liver (Figures 3B and 3C). Immunohistochemical analysis indicated that HFARP protein is present in the hepatocytes, although the pattern of immunostaining is heterogeneous (Figure 3D). Thus HFARP is synthesized in the hepatocytes.

HFARP is a secreted glycoprotein

The amino acid sequence of human HFARP has a highly hydrophobic region at the N-terminus ($\approx$ 23 amino acids) that is typical of a signal sequence for protein secretion (Figure 1A). The signal-sequence cleavage site was predicted to lie between amino acid 23 (Ala) and 24 (Gln) using the method of von Heijne [18]. To demonstrate that HFARP is a secreted protein, COS-7 cells were transfected with CMV promoter-driven mammalian cell expression vector containing human HFARP cDNA with a 3′-terminal extension encoding a *c*-Myc tag and His_6 tag. To detect the HFARP protein, both the culture medium and cell lysates were examined by Western-blot analysis with an anti-Myc antibody. A major HFARP band of $\approx$ 58 kDa was detected in the culture medium, but was barely present in the cell lysate (Figure 4A). The observed molecular mass of the major HFARP band was larger than its calculated molecular mass (45.3 kDa). The amino acid sequence of HFARP contained one potential glycosylation site (amino acid 177) (Figure 1A), suggesting that HFARP, like Ang1, may be a glycoprotein. Therefore the recombinant HFARP was treated with PNGase-F (Figure 4B). Deglycosylation reduced the apparent molecular mass of recombinant HFARP from $\approx$ 58 kDa to the predicted 45.3 kDa (Figure 4B). These results indicate that our recombinant HFARP from transfected COS-7 cells is an efficiently secreted glycoprotein. Thus HFARP protein could be secreted from hepatocytes and be present in the circulating blood. We examined the presence of HFARP protein in the plasma of normal rat. Western-blot analysis indicated that HFARP protein is present in the plasma (Figure 4C).

HFARP prevents apoptosis but does not induce DNA synthesis or sprout formation in vascular endothelial cells

Since HFARP belongs to angiopoietin or fibrinogen family protein, we compared the function of HFARP with Ang1 or fibrinogen in endothelial cells. The inability of Ang1 to stimulate endothelial-cell proliferation has been demonstrated [1,4,9]. We examined the ability of HFARP to stimulate endothelial-cell proliferation by analysing the incorporation of [^{3}H]thymidine into HUVECs. Ang1 (200 ng/ml) or HFARP (200 and 800 ng/ml) did not alter [^{3}H]thymidine incorporation into DNA of HUVECs, whereas $VEGF_{165}$ (5 ng/ml) increased [^{3}H]thymidine incorporation into DNA approx. 2.6-fold (Figure 5A). These results demonstrate that HFARP, like Ang1, is not an endothelial-cell growth factor *in vitro*. Ang1 is an effective inducer of sprouting in endothelial cells *in vitro* [10,11]. Therefore we examined the sprouting activity of HFARP in PPAECs, using $VEGF_{165}$ and Ang1 as positive controls (Figure 5B). Whereas the control buffer produced a basal sprouting activity [approximately six sprouts per 50 microcarrier (MC) beads], $VEGF_{165}$ (10 ng/ml) and Ang1 (200 ng/ml) produced 9.2 and 5.2-fold increases, and HFARP (200 and 800 ng/ml) did not change a sprouting activity, respectively. Ang1 is an apoptosis survival factor in endothelial cells [11,19,20]. We used the serum-deprivation method for inducing apoptosis in HUVECs. The percentage of apoptotic cells increased from 1.2% before serum deprivation to 28.1% at 24 h after serum deprivation (Figures 5C and 5D). Similar to our previous report [11], Ang1 (200 ng/ml) inhibited approx. 50–55% of the apoptotic events that occurred in the control conditions. Similarly, HFARP (200 and 800 ng/ml) inhibited approx. 30 and 45% of the apoptotic events that occurred under control conditions (Figures 5C and 5D). However, fibrinogen (0.1–100 μg/ml) did not have a significant anti-apoptotic effect. In addition, HFARP did not produce a significant antiapoptotic effect on non-endothelial cells, including cardiac fibroblasts, vascular smooth-muscle cells, renal mesangial cells, HeLa cells, and HepG2 cells (results not shown).

HFARP does not bind to Tie1 or Tie2

Ang1 and Ang2 bind to the Tie2 receptor with similar affinity, but neither binds to the closely related receptor Tie1 [1,2]. We assayed the binding activity of HFARP with Tie1 and Tie2 by *in vitro* protein–protein interaction. The secreted extracellular domain of Tie1 or Tie2, pFLAG-eTie1 ($\approx$ 96 kDa) or pFLAG-eTie2 ($\approx$ 105 kDa) respectively was bound to an anti-FLAG

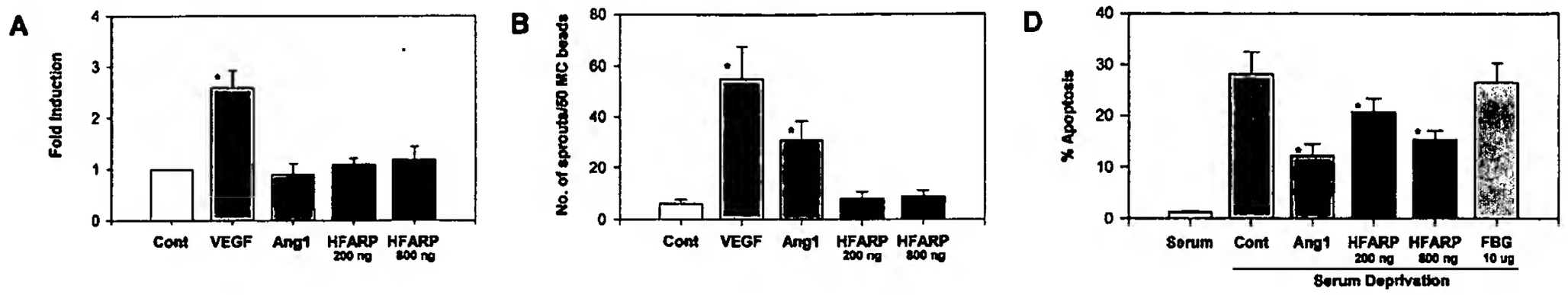


Figure 5 Assays of recombinant HFARP activity in endothelial cells

(**A**) The effect of recombinant proteins on [^{3}H]thymidine incorporation into endothelial cells. HUVECs were incubated with control buffer (Cont), $VEGF_{165}$ (5 ng/ml), Ang1 (200 ng/ml), HFARP (200 and 800 ng/ml), and incorporation of [^{3}H]thymidine was measured. Bars represent the mean $\pm$ S.D. of the fold induction of [^{3}H]thymidine incorporation compared with the control from four experiments. (**B**) The effect of recombinant proteins on sprouting activity in PPAECs. Cells grown on MC beads were placed in fibrin gels containing control buffer, recombinant $VEGF_{165}$ (10 ng/ml), Ang1 (200 ng/ml), or HFARP (200 and 800 ng/ml) protein, and were incubated in M-199 medium with daily supplementation with the same amount of recombinant protein. After 2 days, the extent of sprouting was determined using a phase-contrast inverted microscope. The number of endothelial sprouts with a length exceeding the diameter (175 μm) of the MC bead was determined for every 50 MC beads counted, as described in [9]. Bars represent the mean $\pm$ S.D. from four experiments. (**C**) Effect of recombinant proteins on anti-apoptotic activity in HUVECs. HUVECs were grown for 24 h in the presence of 10% serum and then incubated for 24 h in same medium, or serum-free medium with control buffer (Control), Ang1 (200 ng/ml), HFARP (200 and 800 ng/ml) or fibrinogen (FBG, 10 μg/ml). (a) Phase-contrast microscopy. Note that there are fewer adherent cells and more floating dead cells at 24 h after serum deprivation. The cells exposed to Ang1 or HFARP are more adherent than the cells exposed to control buffer or fibrinogen. Magnifications are 164 $\times$. (b) Light microscopy of TUNEL assay. Arrowheads indicate brown adherent apoptotic cells with fragmented or condensed DNA. Magnifications are 327 $\times$. (**D**) Quantification of apoptotic cells. Percentage of apoptotic cells is based on the sum of the floating cells plus the apoptotic adherent cells in a given cell population. Bars represent the mean $\pm$ S.D. from four experiments. All statistical analysis between the control and experiment was performed using one-way analysis of variance, followed by the Student–Newman–Keuls test. $^*P < 0.05$ versus control.

affinity gel (Figure 6A). As shown in Figure 6(B), recombinant Ang1 bound to pFLAG-eTie2, but not to pFLAG-eTie1, consistent with previous results [1,2]. However, HFARP did not bind to pFLAG-eTie2 or pFLAG-eTie1 (Figure 6C).

DISCUSSION

Angiopoietin family proteins have characteristic protein structures that contain coiled-coil domains in the N-terminal portion and fibrinogen-like domains in the C-terminal portion [1,2,5]. We have isolated a cDNA encoding a novel fibrinogen/angiopoietin-related protein from human and mouse embryonic cDNAs and have designated it HFARP, since its expression is liver-specific. The deduced amino acid sequences of human and mouse HFARP reveal that the N-terminal and C-terminal portions of the protein also contain characteristic coiled-coil domains and fibrinogen-like domains respectively. However, HFARP is not an angiopoietin family protein because HFARP shows a low sequence identity with Ang1 and Ang2 in the N-terminal portion and HFARP does not bind to either Tie1 or Tie2. According to analyses with the BlastP (NCBI), ClustalW and ProteinPrediction (EMBL) programs, HFARP is a member of the fibrinogen superfamily, which includes the fibrinogens, angiopoietins and ficolins. We have named this protein as an hepatic fibrinogen/angiopoietin-related protein for the following reasons: (1) the N-terminal portion of HFARP has coiled-coil domains, like Ang1 and Ang2; (2) its expression is hepatic specific; (3) the neighbour-joining analysis indicates that this

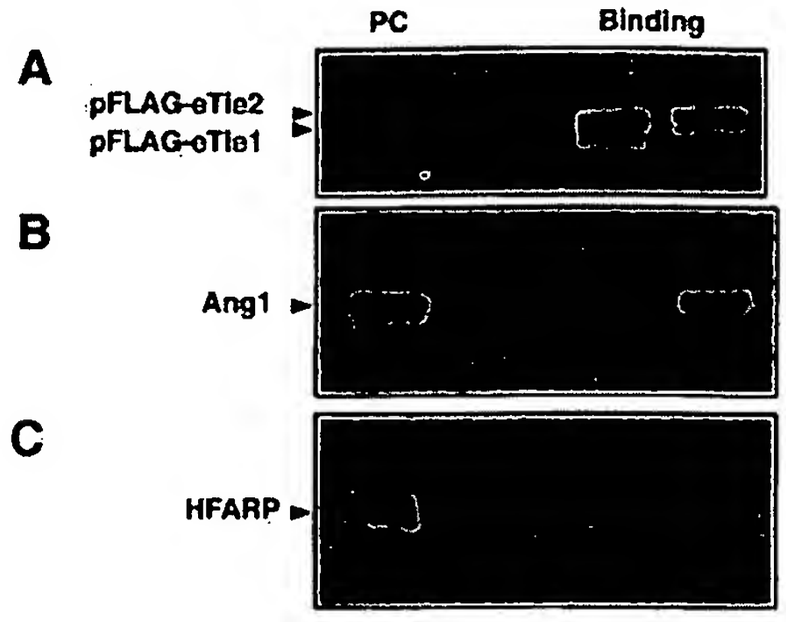


Figure 6 ***In vitro*** **protein–protein interaction between Ang1 or HFARP and pFLAG-eTie1 or pFLAG-eTie2**

(**A**) Control experiment shows that the pFLAG proteins bind to the anti-FLAG affinity gel. After binding of pFLAG-eTie1 or pFLAG-eTie2, the gels were boiled with sample buffer, separated by SDS/PAGE, and probed with anti-FLAG antibody. Note that an equal amount of pFLAG-eTie1 or pFLAG-eTie2 protein is present in the gel. (**B** and **C**) After binding of Ang1 or HFARP to pFLAG-eTie1- or pFLAG-eTie2-coupled gels, the gels were boiled with sample buffer, separated by SDS/PAGE, and probed with anti-Myc antibody. PC, positive control of each recombinant protein (200 ng). Note that Ang1 binds to pFLAG-eTie2, but not to pFLAG-eTie1, while HFARP does not bind to either protein. Results were similar in three independent experiments.

protein is a member of the fibrinogen superfamily; and (4) this protein, like Ang1, is an apoptosis survival factor for endothelial cells.

The expression patterns and the deduced amino acid sequences of the ARP genes shed some light on their potential functions. ARP1 mRNA is also widely expressed in the adult tissues, but it is expressed abundantly in highly vascularized glandular tissues, including the adrenal and thyroid glands [4]. ARP2 mRNA is widely expressed in adult tissues, but is particularly abundant in adult muscle tissues including heart, small intestine and stomach [6]. ARP2 mRNA was preferentially expressed in the blood vessels and skeletal muscles of developing rat embryos [6]. In contrast, HFARP mRNA is exclusively expressed in the liver of embryonic rat and adult mouse. Noticeably, HFARP mRNA is present specifically in the hepatocytes of liver. Like the angiopoietins, HFARP has a highly hydrophobic region at the N-terminus ($\approx$ 23 amino acids) that is typical of a signal sequence for protein secretion. The analysis and comparison of hydrophobicity profiles among Ang1, Ang2, and HFARP were performed using the Kyte and Doolittle algorithm [21]. These results indicated that HFARP, like Ang1 and Ang2, mainly consists of hydrophilic amino acids (results not shown), and is likely to be a secreted protein. Indeed, COS-7 cells transfected with CMV promoter-driven mammalian cell expression vector containing human HFARP cDNA produced a major HFARP protein band of $\approx$ 58 kDa in the culture supernatant, but not in the cell lysate. Deglycosylation reduced the apparent molecular mass of the secreted recombinant HFARP from $\approx$ 58 kDa to the 45 kDa predicted by the sequence. Therefore HFARP could be synthesized in hepatocytes and secreted into the blood. In fact, Western-blot analysis indicated that HFARP protein is present in the circulating blood in detectable amounts. These results indicate that HFARP is likely to be a glycoprotein efficiently secreted from the liver.

The Tie family of receptors consists of two members, Tie1 and Tie2 or TEK [22]. Tie1 and Tie2 are single transmembrane receptor tyrosine kinases. Tie1 and Tie2 are expressed predominantly in vascular endothelial cells and some haematopoietic cells [23,24]. The studies on mice with a targeted disruption of the *Tie1* or *Tie2* gene suggest that the Tie1 signals control fluid exchange across capillaries and haemodynamic resistance [25,26] and Tie2 may control the ability of endothelial cells to recruit stromal cells to encase the endothelial tubes to stabilize the structure and modulate the function of blood vessels. Ang1 and Ang2 are ligands for Tie2 and bind to it with similar affinity [1,2]. The study of transgenic overexpression reveals that Ang2 is a naturally occurring antagonist of Ang1. Ang2 competes for Ang1 binding to Tie2 and blocks Ang1-induced Tie2 autophosphorylation [2]. Both Ang3 and Ang4 bind to Tie2 but not to Tie1 [5]. Moreover, Ang4, like Ang1, is an agonist for the Tie2 receptor, whereas Ang3, like Ang2, is an antagonist for Tie2 [5]. However, the recently discovered ARP1 and ARP2 do not bind to Tie2 and Tie1 [6]. Therefore, to date, ligands for Tie1 have not been identified. Given the similarity between the angiopoietins and HFARP, we speculated that HFARP could be a ligand for Tie1 or Tie2. Therefore we assayed the binding activity of HFARP to Tie1 and Tie2 using the secreted extracellular domains of the Tie1 and Tie2 receptors. However, HFARP does not bind to the Tie1 or Tie2 receptor.

Given that HFARP is a circulating protein that is secreted from liver, we considered the effect of HFARP on endothelial cells. Although HFARP does not have effects on DNA synthesis or sprouting in endothelial cells, HFARP has a significant anti-apoptotic effect in endothelial cells mediated through an unidentified receptor or receptors. Ang1 exerts similar anti-apoptotic actions [11,19,20], whereas fibrinogen does not exert anti-apoptotic effects in endothelial cells. Vascular endothelial cells directly contact the plasma and the cellular components of the blood, and, under certain pathological conditions, are the apoptotic targets of various noxious stimuli, such as toxins, drugs and physical agents [27]. Under these situations, HFARP may be an important circulating molecule for protecting endothelial cells from a damage through endocrine action.

In summary, we have isolated a novel fibrinogen/angiopoietin-related protein and have designated it HFARP, since its expression is liver-specific. HFARP contains the characteristic coiled-coil domains and fibrinogen-like doins found in angiopoietins, but has only modest overall sequence similarity to them. HFARP mRNA and protein are mainly present in the hepatocytes. HFARP is a glycosylated secretory protein that is an apoptosis survival factor for endothelial cells through an endocrine action.

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